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Molecular Imaging of Proteolytic Activity in Cancer

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Abstract The early detection of both primary tumors and metastatic disease continue to be significant challenges in the diagnosis and staging of cancer. The growing recognition of the role of proteinases and proteolytic cascades in both the growth and metastasis of tumors has led to the development not only of therapeutic strategies using proteinase inhibitors, but also of methods to detect and image tumors in vivo via tumor-associated proteolytic activities. These imaging strategies derive from the enhanced sensitivity afforded by amplification that can be obtained by enzymatic processing to increase the efficacy of imaging ''contrast agents'' coupled with the inherent substrate specificity and selectivity of proteinases. This review describes key proteinases important in cancer progression, the strategies that have been devised to detect and image proteolytic activity in vivo, and the potential for this kind of functional imaging to serve as a marker for targeted therapy. The intent is to draw attention to the developing methods of molecular imaging to facilitate not only cancer diagnosis, but also for devising strategies for individualized targeted therapy and non-invasive monitoring of therapeutic efficacy. J. Cell. Biochem. 90: 1087–1097, 2003.

Key words: optical imaging; proteinase; MMP–7

Proteinase activities are essential for a number of physiological processes including digestion, hemostasis, wound healing, and tissue remodeling. In many of these processes, the proteinases mediate and/or regulate both intercellular signaling, such as in the release and/or processing of chemokines, and intra-cellular pathways such as in the apoptotic pathways leading to programmed cell death. Disregulation of the temporal and/or spatial co-ordination of these intra-cellular and/or inter-cellular pathways disrupts the normal physiology and rhythm of life that can be manifest in unregulated growth such as occurs in tumors and their metastatic progeny. The evolving revelation of the diverse range of biological functions of the proteinases in normal growth and development of multi-cellular organisms has been accompanied by recognition of the significance of a variety of proteinases and proteolytic cascades in the pathophysiology of cancers. Both intracellular and extracellular proteinases from each of the five main groups, the aspartic, cysteine, serine, and threonine peptidases as well as the metalloproteinases [Barrett et al., 1998; Rawlings et al., 2002] are now known to either contribute to or are implicated in various aspects of tumor growth, invasion, and metastasis.

INTRACELLULAR AND EXTRACELLULAR PROTEOLYSIS IN CANCER

The life cycle of individual cells involves the co-ordinated turnover of sets of regulatory proteins that require timely and irreversible degradation for progression through the various life stages and that ultimately end in programmed cell death. Such protein degradation is mediated by the ubiquitination-proteasome pathway with the proteosome (a threonine proteinase) being the dominant proteinase dedicated to protein turnover [Bogyo and Wang, 2002]. Studies of animal models have identified a number of potential target proteins that appear to play a role in the growth of breast carcinomas and either regulate or are regulated by the ubiquitin-proteosome system [Rossi and Loda, 2002] with at least one proteosome inhibitor having been proposed for treating

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chemoresistant tumors [Cusack, 2003]. Intracellular proteinases of the caspase family of cysteine-dependent aspartate-specific proteinases have also been implicated in the growth and metastasis of tumors via their roles in the apoptotic pathways leading to programmed cell death [Kiechle and Zhang, 2002; Salvesen, 2002], a process that is an essential part of normal tissue homeostasis. Disregulation in one or more steps of the caspase-mediated apoptotic signaling pathways has been demonstrated in both melanoma and lymphoma [Ivanov et al., 2003; Rossi and Gaidano, 2003]. In such cases, tumorigenesis appears to result from an impaired ability to undergo programmed cell death in response to external stimuli, reflecting a lack of activation of the apoptotic pathway. This kind of loss of apoptotic signaling in tumors has promulgated the development of therapeutic strategies aimed at stimulating apoptosis via the activation of the caspases that are important components of these pathways [Kiechle and Zhang, 2002].

Cancers that progress to metastatic disease have a poor prognosis and are life-threatening. A critical step in this pathological pathway to metastasis is the breaching of the basement membrane that permits escape of cells from a primary tumor into the circulation and/or lymphatic system. In this regard, the metastatic process can be viewed as a disregulation of the complex interplay between the cellular components of tissues and their surrounding matrix. In multicellular organisms, such interactions between cells and their environment, including proteins and other components of the matrix, remain poorly understood though it is these kinds of interactions that define not only the composition, but also the size and shape of tissues, organs, and whole organisms. A significant advance in understanding the biochemical and physiological processes involved in such cell–matrix interactions was the discovery (now about 40 years ago) of collagenase (now called collagenase-1 or MMP-1), a proteinase that was involved in the resorption of the tadpole tail during morphogenesis [Gross and Lapiere, 1962]. Collagenase is now recognized as an matrix metalloproteinase (MMP), a family of extracellular, zinc-dependent proteinases capable of degrading all components of the extracellular matrix ([Woessner and Nagase, 2000; Brinckerhoff and Matrisian, 2002] for reviews) that, in humans, constitute 24 distinct gene products. Lance Liotta's pioneering work in the late 1970s [Liotta et al., 1980] indicated that the degradation of collagen in the basement membrane is an important component of tumor invasion and metastasis. It is now recognized that the MMPs together with some members of two other families of metalloproteinases, the ADAMS (a disintegrin and metalloproteinase) and ADAMTS (ADAM with thrombospondin motif) with a total of more than 50 members [Duffy et al., 2003; Seals and Courtneidge, 2003; Somerville et al., 2003a,b], participate in extracellular proteolysis of the polymeric/fibrillar and non-fibrillar matrix proteins as well as non-matrix proteins [McCawley and Matrisian, 2001]. Notably, a number of the MMPs ([Nelson et al., 2000; Brinckerhoff and Matrisian, 2002] for reviews) are families of degradative enzymes with clear links to malignancy. The cathepsins, lysosomal enzymes of the cysteine or aspartic acid class, have also been implicated in matrix degradation and tumor progression [Herszenyi et al., 2000; Koblinski et al., 2000; Lecaille et al., 2002]. Cathepsin K is produced by osteoclasts and degrades bone matrix components in specialized extracellular acidic compartments [Bromme and Kaleta, 2002; Lecaille et al., 2002]. Cathepsin D has long been associated with breast cancer progression [Duffy, 1996; Rochefort et al., 2000]. The cysteine proteinase, cathepsin B, is expressed in a number of steps in malignant progression being implicated in tumor–stromal interactions and matrix degradation as well as neovascularization and angiogenesis [Koblinski et al., 2000]. Likewise, the serine proteinases, particularly those of the S1 or trypsin-like family that contribute to normal homeostasis, have been implicated in pathological processes including cancer [Netzel-Arnett et al., 2003]. Notably, the plasminogen/plasmin system participates in tissue remodeling and extracellular matrix degradation and is one of the main proteolytic cascades involved in tumor cell invasion and metastasis [Berger, 2002]. For example, the urokinase-type plasminogen activator (UPA), a serine proteinase in the plasminogen/plasmin pathway has been implicated in gastrointestinal neoplastic disease [Herszenyi et al., 2000]. Studies have shown that the plasma levels of UPA, as well as the serum levels of the cysteine proteinase cathepsin B, are significantly increased in patients with gastrointestinal tumors [Herszenyi et al., 2000; Nijziel et al., 2003]. Other serine proteinases

implicated in cancer include the more recently identified membrane-anchored proteinases that appear to perform complex regulatory cellular signaling functions both at the plasma membrane and within the extracellular matrix but exhibit disregulation in tumors [Netzel-Arnett et al., 2003]. In particular, a group of type II integral membrane proteinases, including seprase, that interact with a variety of membrane-associated molecules and substrates, appear to localize at cell surface protrusions called invadopodia and play a prominent role in cell migration and matrix invasion, processes that are essential for tumor invasion, angiogenesis and metastasis [Chen and Kelly, 2003]. For many of these proteinases, the activation process is mediated by a proteolytic cascade, e.g., plasmin and stromelysin-1 (MMP-3) cooperate to produce fully activated collagenase from procollagenase [Brinckerhoff and Matrisian, 2002]. Based on the co-localization of the serine proteinases, MMPs, and cathepsin B, it has been postulated that these kinds of tumor-associated extracellular proteinases participate in proteolytic cascades on the tumor cell surface [Brinckerhoff and Matrisian, 2002; Roshy et al., 2003] that may also contribute to the pathophysiology of disease progression in cancer.

IMAGING MODALITIES USED IN CANCER DETECTION

A variety of imaging modalities are used for the clinical detection and imaging of both primary tumors and metastatic disease. With standard protocols, these imaging methods detect tumors via anatomical parameters or tumor vascularity rather than specific biochemical or physiological characteristics. However, such non-invasive imaging techniques provide an extraordinary opportunity for "molecular imaging" of tumors to increase the sensitivity of detecting early-stage tumors, to identify tumors that require particularly aggressive therapy, to identify tumors appropriate for specific anti-proteinase therapeutics, and to address the issue of target modulation and dose selection [Hoffman, 2000]. There have been major advances in whole animal molecular imaging that are no less dramatic than the remarkable advances in optical imaging at the cellular and molecular level that have been driven for the most part by the introduction of new fluorescent probes for real-time imaging of biochemical processes in live cells [Zhang et al., 2002]. While cell imaging relies primarily on optical methods, molecular imaging in whole animals has been achieved using a variety of non-invasive imaging modalities including magnetic resonance (MR) [Evelhoch, 1999; Choyke et al., 2003; Gore, 2003; Harisinghani et al., 2003; Nelson, 2003; Raghunand et al., 2003; Ross et al., 2003], nuclear [Ritman, 2002; Rowland et al., 2002; Blasberg, 2003; Herschman, 2003], and optical [Gulsen et al., 2002; Smith, 2002; Edinger et al., 2003; Mahmood and Weissleder, 2003]. The power of these kinds of techniques has been amplified by the development of reagents and protocols aimed at detecting and imaging specific cellular and molecular processes in vivo. Many of these novel imaging strategies include the use of biochemically activated or targeted "contrast agents" designed to detect metabolic and/or biochemical differences between tumors and their surrounding host environment. For example, detection and imaging of cancer has been achieved in pre-clinical studies with novel optical imaging strategies using either bioluminescence [Mandl et al., 2002; Edinger et al., 2003] or fluorescence based on either intrinsic differences in auto-fluorescence of tissues such as those in the aural cavity [Smith, 2002] or from studies following the administration of fluorogenic substrates [Bremer et al., 2003; Mahmood and Weissleder, 2003]. Likewise, recent technological innovations in both MRI and radionuclide-based imaging modalities such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), have led to the development of new methods for the clinical detection and imaging of both primary tumors and metastatic disease (see e.g., [Blasberg, 2003; Herschman, 2003; Ross et al., 2003] for reviews). These developments in molecular imaging are beginning to revolutionize the practice of diagnostic radiology from an anatomical discipline to one increasingly based on functional profiles [Gillies, 2003]. The advantage of these kinds of imaging methods is that they can be applied to give both temporal and spatial dimensions to characterize the progression of and/or treatment of specific disease processes. In addition, molecular imaging has the potential not only to enhance the detection of tumors but also for developing patient-specific treatment strategies based on the in vivo biochemical pathology of specific tumor-associated processes.

IMAGING OF PROTEOLYTIC ACTIVITY IN CANCER

The tumor-associated proteinases present attractive targets for in vivo detection and molecular imaging from a number of perspectives. Firstly, a number of the proteinases are secreted and active in the extracellular matrix where they are more readily accessible to substrates delivered through the vasculature (or lymphatic system), without requiring specific targeting of reagents to tumor cells. Secondly, the enzymatic activity can be utilized as a signal amplification system whereby signal (contrast) can be enhanced over time by cleavage of multiple substrate molecules via the intrinsic activity of the proteinase. Thirdly, a number of the extracellular proteinases including the MMPS and cathepsins are expressed only in a limited set of physiological or pathological processes [Koblinski et al., 2000; Nelson et al., 2000]. The detection and imaging of proteolytic activity in vivo has been achieved not only with extracellular proteinases like the cathepsins and MMPs but also for caspase-3, an intracellular proteinase that participates in one of the pathways to programmed cell death.

The in vivo optical detection and imaging of proteinase activity was first demonstrated less than 5 years ago [Weissleder et al., 1999] with the report of in vivo imaging of mouse xenograft tumors that was thought to be mediated by tumor-associated lysosomal proteinase activity. The optical contrast agents developed for such studies used near infra-red (NIR) fluorophores as optical sensors attached to an essentially linear poly-lysine-polyethyleneglycol copolymer that was injected into tumor-bearing mice. The proximity of the fluorophores on the polymer substrate quenched the fluorescent signal, which was then enhanced by proteolytic cleavage of the peptide linker, producing an optically detected near infrared fluorescence (NIRF) signal associated with the tumor [Weissleder et al., 1999]. Subsequent modifications to the fluorogenic copolymer substrate probe, e.g., by linking the NIRF sensor to the polymer via a peptide containing a proteolytic cleavage site, have permitted detection of proteolytic activity in a variety of disease settings [Weissleder and Mahmood, 2001; Weissleder, 2002; Bremer et al., 2003]. For example, Cy 5.5-based reagents containing a peptide with some selectivity for cathepsin B have been used for the in situ detection of intestinal adenomas [Marten et al., 2002] via the detection of adenoma-associated cathepsin B activity in the Min mouse model of adenomatous polyposis. A similar reagent has been used in studies to distinguish well-differentiated and undifferentiated mammary tumors in a mouse model of human breast cancer [Bremer et al., 2002], demonstrating the utility of this approach not only for cancer detection, but also for diagnosis and staging of disease progression. Results from those studies hold promise for the use of these kinds of optical contrast agents for detecting and imaging tumor-associated proteolytic activity in vivo with a prospect for identifying pre-cancerous lesions using "bioendoscopy," a new tool in an anticipated revolution in gastrointestinal imaging [Pasricha and Motamedi, 2002], or by optical tomography, another emerging method for whole-body imaging of NIRF probes [Mahmood and Weissleder, 2003].

Intracellular proteinases, exemplified by caspase-3 in the apoptotic pathway, have also been optically imaged in vivo by using a luciferase reporter gene and bioluminescence [Laxman et al., 2002]. In these studies, a reporter gene was constructed so as to produce a caspase-3 cleavable recombinant luciferase fusion protein in which the activity of the luciferase reporter was silenced by fusion with the estrogen receptor regulatory domain coupled to luciferase via a caspase-3 cleavable linker [Laxman et al., 2002]. This silenced luciferase was introduced into glioma cells and was shown to be responsive and activated by caspase-3 during apoptosis induced either in vitro or in subcutaneous xenografts after treatment with tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL). The activation of caspase-3 could be visualized by bioluminescence imaging of the activated luciferase yielding real-time in vivo imaging of apoptosis. This kind of imaging technique provides an approach to study the role of apoptosis in various disease processes as well as to evaluate experimental therapeutic agents directed at the apoptotic pathway [Laxman et al., 2002]. The in vivo detection and imaging of apoptosis has also been demonstrated using derivatives of annexin-V labeled either with a radioactive element [Narula et al., 2001] or with a NIRF-tag [Petrovsky et al., 2003]. Thus, apoptosis can be detected and imaged in vivo both via the binding of annexin-V

that detects the externalization of aminophospholipids [Petrovsky et al., 2003] and by caspase-3 activation [Laxman et al., 2002]. The use of the kind of reporter gene developed to detect caspase-3 activity in vivo has great potential for studying specific proteinases in pre-clinical disease models.

Proteinases including MMPs are generally expressed and secreted as zymogens that are latent pro-forms of such enzymes that require proteolytic processing to their mature catalytically active form. Thus, detection of expression does not necessarily correspond with proteolytic activity. While the silenced reporter described above to detect and image apoptosis uses luciferase activity as a direct detector of proteolytic activity in vivo, other strategies have been developed to image proteases indirectly. For example, Davidson and colleagues have used a modified luciferase reporter gene and bioluminescence imaging to achieve real-time in vivo visualization of the promoter activity of the collagenase MMP-13 during cutaneous wound repair in mice [Wu et al., 2002]. In those studies, transgenic mouse lines were generated with the luciferase reporter gene under control of the MMP-13 promoter with MMP-13 promoter activity being visualized and quantified in vivo by bioluminescence. The results indicated that MMP-13 participates in healing of acute wounds and is a significant factor in the long-term remodeling of wound connective tissue in rodent skin [Wu et al., 2002]. By contrast with the apoptosis studies that used an activable luciferase as substrate to directly detect caspase-3 activity [Laxman et al., 2002], the measurement of MMP-13 promoter activity provides a direct assessment of expression but only an indirect measure of proteolytic activity presumed to result from induction of the MMP-13 promoter. Recent optical studies report a novel method for the indirect in vivo imaging of MMP activities via the in vivo imaging of collagen or extracellular matrix using second harmonic generation (SHG) either alone [Brown et al., 2003] or together with two-photon fluorescence [Zoumi et al., 2002]. The characterization of the in vivo structure of tumor-associated collagen or extra-cellular matrix from SHG images could be interpreted in terms of an increase in activity of soluble MMPs accompanied by an upregulation of matrix production in tumors with an attendant increase in porosity of tumor-associated collagen matrix [Brown et al., 2003]. By using back-scatter imaging of collagen fibers together with immunofluorescent detection of specific proteinases, Friedl and colleagues have been able to characterize the role of extra-cellular proteinases in tumor cell invasion and migration via real-time imaging of cells on collagen matrices using techniques that may also be applied to in vivo imaging of skin and epithelia [Friedl and Wolf, 2003; Wolf et al., 2003]. We are not aware of studies that use MRI to detect proteinase activity in vivo, though Meade and colleagues have devised a novel strategy to image beta-galactosidase gene expression by MRI using a galactosidase-sensing MRI contrast agent [Louie et al., 2000]. These studies together with the recent report of superparamagnetic nanoparticles that serve as magnetic relaxation switches to detect proteinase activity either in vitro or with cultured cells [Zhao et al., 2003] indicate the potential of MRI for detecting and imaging tumor-associated proteolytic activity in vivo.

There is the potential that the development of imaging methodologies for the detection of proteolytic activity could be refined to develop highly specific or selective monitors of the activity of individual proteinases. A wealth of information has been generated regarding the association of specific proteinase family members with clinically-relevant parameters of tumor progression. For example, elevated serum levels of prostate-specific antigen (PSA), an androgen-regulated serine proteinase in the kallikrein family, has long served as a biomarker both to detect prostate cancer and to assess treatment efficacy [Corthals and Nelson, 2001; Balk et al., 2003]. Likewise, high UPA expression in breast cancer correlates with poor prognosis and low levels serve as a reliable marker to identify low-risk node-negative breast cancer patients for whom adjuvant chemotherapy is unnecessary [Duffy, 2002]. In this context, UPA expression may be a more reliable prognostic marker than the long-established elevated levels of estrogen receptor that generally predicts favorable disease outcome [Fuqua, 2001] though may also indicate a poor prognosis later in the disease process [Speirs, 2002]. In the MMP family, the expression of MMP-11 in breast cancer has been associated with malignant disease, and it is not expressed in normal breast tissue or benign fibroadenomas [Wolf et al., 1993]. In melanomas, the expression of

MMP-9 is associated with the conversion from radial growth phase to vertical growth phase and subsequent metastasis [MacDougall et al., 1995]. MMP-7 expression is associated with poor outcome in esophageal, colon, and pancreatic cancers [Yamamoto et al., 1999; Adachi et al., 2001; Yamamoto et al., 2001]. Thus, the expression of proteinases has been shown to be useful in distinguishing benign from malignant tumors, or in identifying aggressive tumors associated with poor outcome. This information has been generated from biopsies or resected tumor specimens, requiring invasive procedures. An exciting aspect of the potential for selectively imaging different proteinases in vivo is the prospect for developing contrast agents that will allow the non-invasive assessment of MMP expression associated with specific tumors. In the clinical setting, this kind of diagnostic molecular imaging would have the potential to rapidly provide important information on disease status and serve to guide treatment decisions for individual patients. The in vivo imaging of specific MMP activities has the potential to provide non-invasive assessment of proteinase profiles that might then be related to clinically relevant parameters of tumor progression.

IMAGING OF PROTEOLYTIC ACTIVITY TO MONITOR PROTEINASE-TARGETED THERAPEUTICS

MMPs have long been recognized as potential anti-cancer targets for which a number of both general and specific inhibitors (MMPI) have been developed (see [Sternlicht and Bergers, 2000; Coussens et al., 2002], for reviews). However, a record of positive results in preclinical trials of MMPIs was followed by disappointing negative results in Phase III clinical trials. The discrepancy appears to reside in a number of unresolved issues related to the type and stage of cancers that were examined, and importantly, to the specificity, dose and toxicity of the MMPIs (see [Fingleton, 2003], for review). A major issue is distinguishing between the failure of the MMPI to effectively inhibit tumor MMP activity at the administered dose versus a failure of inhibition of MMP activity to impact patient survival or time-to-progression. In vivo imaging of tumor-associated proteinase activities has the potential for longitudinally monitoring the in vivo efficacy of proteinase-targeted therapeutic strategies, providing a tool both to select patients for particular therapeutic protocols as well as for establishing the optimal dose to achieve in vivo inhibition of a selected proteinase target.

In pre-clinical studies, this potential has already been fulfilled via the detection and imaging of tumor-associated proteolytic activity in HT1080 human fibrosarcoma xenografts by using a NIRF-sensor containing a peptide sequence designed to detect MMP-2 activity [Bremer et al., 2001]. In those studies, the NIRF-substrate was linked to a non-immunogenic graft co-polymer designed as an efficient delivery vehicle. This polymer substrate was shown to be cleavable in vitro by MMP-2 although an increase in fluorescence could also be detected after treatment with a number of other MMPs. In vivo studies showed selective detection of xenograft tumors expressing MMP-2 that gave ~4-fold higher NIRF than control tumors following intra-venous injection of the NIRF-proteinase substrate. Notably, the treatment of tumor-bearing mice with the broad spectrum MMPI, prinomastat, markedly reduced the tumor-associated fluorescence to ~40% of that in the xenograft tumors of untreated animals indicative of in vivo proteinase inhibition by this synthetic MMPI [Bremer et al., 2001]. Those studies were the first demonstration that MMP inhibition could be imaged in vivo after administration of a potentially therapeutic dose of MMPI.

IMAGING OF MMP-7 ACTIVITY AND ITS INHIBITION

Studies in our laboratory have focused on the role of MMP-7 in tumor growth and metastasis. MMP-7 is biochemically distinct from most of the MMPs , being one of only two MMPs that contain only the catalytic domain in its active form (reviewed in [Wilson and Matrisian, 1998]). Of particular interest, it is expressed predominantly in cells of epithelial origin, in contrast to other MMPs whose expression is often localized to connective tissue. Thus, in adenocarcinomas (the most common adult tumor types that arise from cells of epithelial origin), MMP-7 is expressed in the malignant epithelial component of the tumor rather than in the surrounding stromal tissue. From a functional imaging perspective, this provides a focused source of an abundantly-expressed

enzyme to facilitate in vivo detection and quantitation. Finally, MMP-7 is one of the few MMP family members whose expression can be detected in benign tumors [Newell et al., 1994], and we have evidence that MMP-7 represents a reasonable target for controlling disease progression in high-risk patients. For example, we have demonstrated that mice that are rendered genetically deficient in MMP-7 develop fewer benign intestinal adenomas in a mouse model of familial adenomatous polyposis [Wilson et al., 1997]. In addition, transgenic mice in which MMP-7 is over-expressed in the mammary epithelium develop hyperplastic nodules and exhibit accelerated and increased incidence of neu-induced mammary tumors [Rudolph-Owen et al., 1998]. Based on this evidence, we suggest that synthetic inhibitors designed to inhibit MMP-7 activity could be used to target early-stage lesions as chemopreventive agents. We have experimental evidence indicating that synthetic MMP inhibitors with efficacy against MMP-7 reduce the number of adenomas in a preclinical (mouse) model of intestinal polyps that supports the feasibility of this approach [Goss et al., 1998]. Thus, we have been developing reagents to detect and image MMP-7 activity in vivo with a view to using these tools both to assess the in vivo efficacy of MMPI in preclinical models of cancer and to guide the development of clinically useful MMPIs.

It is in this context that recent work in our laboratory has been directed towards the detection and imaging of tumors using novel optical contrast agents that we refer to as "Proteolytic Beacons" (PBs) that are designed to be activated by specific MMP family members. The PBs as illustrated in Figure 1, have two features distinct from the MMP reagent developed by the Weissleder group [Bremer et al., 2001] and that provide substantial advantage for imaging tumor-associated MMP activity and the in vivo efficacy of MMPI. Based on our experience with MMPs in cancer, the PBs were designed to be cleaved selectively by MMPs implicated in the growth and/or metastasis of a number of tumors and carcinomas. Thus far, we have prepared and tested PBs that can be selectively cleaved by either MMP-7 or MMP-2, referred to as PB-M7VIS and PB-M2VIS, respectively (see Fig. 1A). The peptide sequence used in Bremer et al. [2001] is a more general substrate for MMPs, as well as for some other proteinases, and thus is highly appropriate for detection purposes but does not provide the specificity desired to distinguish inhibitory effects on enzymes involved in cancer progression versus those involved in normal biological functions such as joint repair. A novel feature of the PBs we designed is the inclusion of an internal reference fluorophore that provides for the detection of both the uncleaved and cleaved reagent. This internal reference facilitates the analysis of proteinase activities both ex vivo and in vivo by providing a measure of the substrate concentration in image-based assays. Thus, the internal reference provides a measure of the ratio of cleaved:uncleaved substrate, a feature that will not only facilitate the analysis of the proposed in vivo imaging studies but also, we believe, will be critical to the successful clinical application of this technology.

The prototype PB, referred to as PB-M7VIS, was built on a dendrimeric polymer core, Starburst® PAMAM, with the fluorescein (FL) optical sensor linked via an MMP-7 selective peptide to the dendrimer scaffold that was also labeled with tetramethylrhodamine (TMR) as an internal reference. Treatment of PB-M7VIS with purified MMP-7 results in a significant enhancement in the FL fluorescence with a minimal change in the fluorescence of TMR (Fig. 1B). MMP activity can therefore be visualized as a green fluorescent signal that is referenced and normalized against the nearly constant red fluorescent signal obtained from the stable TMR-labeled backbone. An in vitro fluorescence assay for MMP activity reveals efficient metalloproteinase-dependent cleavage of PB-M7VIS by MMP-7 and much slower cleavage of PB-M7VIS by either MMP-3 or MMP-2, ~10-fold or ~60-fold, respectively. Thus, PB-M7vis is useful for in vitro measurement of selective MMP-7 activity.

PB-M7VIS has been used to detect MMP-7 positive xenograft tumors in mice. Typical data obtained 2 h after injection of PB-M7VIS are shown in Figure 2. In the green channel, the photon intensity detected from either the control tumor (SW480neo) or the back of the animal is similar and each are comparable to the background signal prior to administration of the reagent (attributed to a combination of backscatter and auto-fluorescence) that averaged in the range from 400 to 500 photons/pixel/10 s for this study (not shown). By contrast, a markedly higher photon flux (~520 photons/pixel/10 s above background, $n = 3$) is detected over the

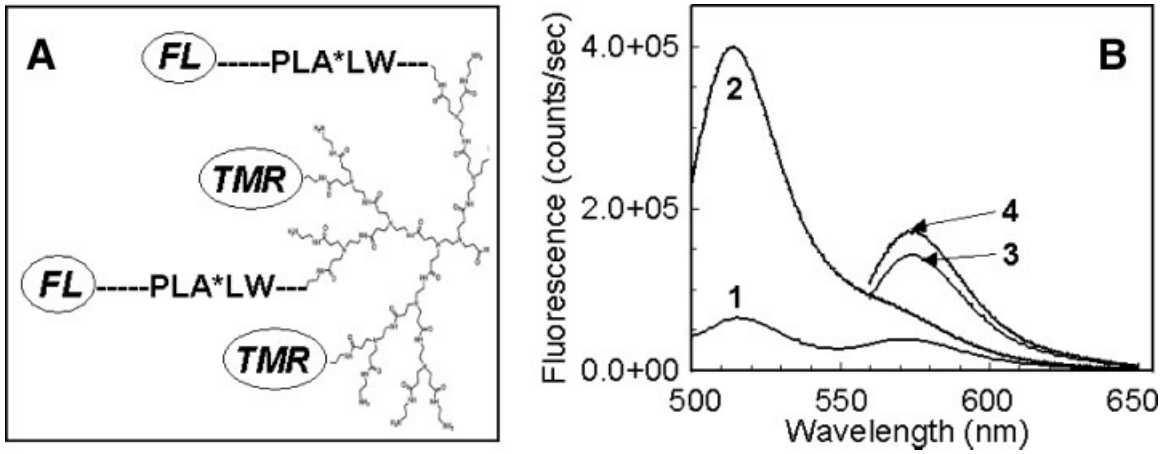

Fig. 1. **A**: Schematic structure of proteolytic beacon PB-M7VIS, designed as a fluorogenic substrate for MMP-7. The fluorescein (Fl) optical sensor is linked via an matrix metalloproteinase (MMP)-selective cleavable peptide to the dendrimeric polymer (depicted in partial structure) also labeled with an internal reference, tetramethylrhodamine (TMR). **B**: Fluorescence emission spectra (Fl, spectra 1 and 2; TMR, spectra 3 and 4) for PB-M7VIS reagent both before (spectra 1 and 3) and after (spectra 2 and 4) treatment with MMP-7.

SW480mat tumor that expresses MMP-7. It is notable that control tumors, produced using SW480 cells, showed fluorescence similar to the background although these cells have been reported to express MMPs-9, -13, -14, and -15 [Giambernardi et al., 1998]. This suggests that the PB-M7VIS reagent is specific for MMP-7 and does not detect these other MMP activities in this tumor setting. In preliminary studies, prior treatment of the mice with the broad spectrum MMPI, BB94, reduced the detected Fl fluorescence over the MMP-7-positive xenograft tumor to ~50% of that detected without MMPI treatment (see Fig. 2, Panel E). Taken together, the fluorescence imaging studies of living mice indicate that PB-M7VIS can be used to detect and image MMP-7 positive xenograft tumors in vivo via the enhanced FL fluorescence of the proteolyzed reagent. Due to both absorption of visible photons, primarily by hemoglobin, and their scattering through tissue, a significant improvement in optical imaging has been predicted by using longer wavelength NIR chromophores [Weissleder, 2001]. In preliminary

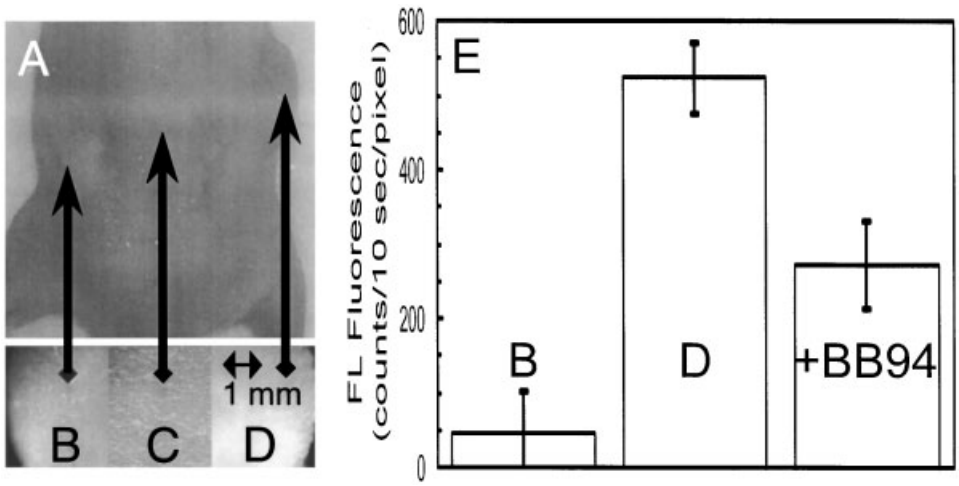

Fig. 2. MMP-7 activity detected in vivo by quantitative Fl fluorescence imaging of mouse SW480neo and SW480mat xenograft tumors (images **B** and **D**, respectively) and a non-tumor region (image **C**), 2 h after intra venous injection of PB-M7VIS. The bar graph (**Panel E**) depicts the average intensity ± the intensity standard deviation in the green channel (n = 3) for tumors B and D and for the SW480mat tumor after treatment of the mouse with BB94, an MMPI (McIntyre et al., 2003).

studies with analogous PBs built with far-red and NIR sensors, we find an ~10-fold improvement in the signal-to-noise ratio compared with PB-M7VIS for in vivo imaging of tumor-associated MMP activity. Such reagents have potential application both for enhancing the detection of tumors via tumor-associated proteolytic activity and for assessing the in vivo efficacy of MMPIs.

FUTURE DIRECTIONS

Non-invasive, in vivo imaging of proteolytic activity has the potential to greatly enhance current medical practice. Optical imaging is currently restricted to easily accessible surface tumors, although the development of optical tomography may extend such studies to detection deeper within tissues [Mahmood and Weissleder, 2003]. The potential to use an endoscopic fluoroscope would facilitate the translation of optical imaging technology to the detection of neoplastic lesions in the epithelial lining of the intestinal and pulmonary tracts. However, the more general utility of this kind of approach may be better served by the development of analogous proteinase-activated MRI proteolytic beacon contrast agents that could be detected and imaged by MRI with equipment already in routine use in clinical practice for the assessment and staging of cancer. Both intra- and extra-cellular tumor-associated proteinases present as potential targets for the detection of tumors by optical and/or MRI imaging modalities, as non-invasive diagnostic and prognostic tools, and as a means to design and monitor appropriate patient-specific targeted therapeutic strategies.

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